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14. ABSTRACT

Androgen receptor (AR)-mediated transcription is modulated by interaction with co-regulatory proteins. We identified a novel AR repressor complex comprising of the androgen receptor co-repressor Art-27 and the transcriptional repressor URI. We found through chromatin immunoprecipitation experiments that URI depletion results in increased H3K9me3 at the promoter of the AR target gene Nkx3.1, suggesting that one mechanism of URI-mediated transcriptional repression is through chromatin modification. Art-27 was also found by ChiP to localize at the transcription start site of Nkx3.1 along with PolII. Previous results demonstrated a direct interaction between URI and the transcription factor KAP1. Because of the tight association of Art-27 with URI, we hypothesized that Art-27 also affects KAP1 activity. Immunoprecipitation of Art-27 with KAP1 revealed a direct interaction between the two proteins. Consistent with this result, knockdown of Art-27 resulted in increased expression of the KAP1 targets LINE1 and AluYa5. Overall, our findings demonstrate a new role for Art-27 in KAP1-mediated gene repression.

15. SUBJECT TERMS
URI; Art-27; AR mediated cell growth: prostate cancer progression

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Table of Contents

INTRODUCTION

The androgen receptor (AR) is the master regulator of prostate cell proliferation, differentiation, and survival. The key role of AR is still maintained in prostate cancer cells even after the development of the castration-resistant stage of the disease (Chen et al., 2004; Feldman and Feldman, 2001). These observations underscore the importance of understanding the molecular mechanisms regulating AR-mediated transcription. Cofactors, co-repressors, and co-activators are responsible for the regulation of AR-mediated transcription, and therefore their mis-regulation or aberrant expression can impact tumor formation and progression. We previously identified a novel AR repression complex consisting of AR and the cofactors URI and Art-27. We also validated in the 2012 report the interaction between URI and the transcription factor KAP1. KAP1 has been shown to repress genes involved in apoptosis and retroelement expression by recruiting histone methyltransferases such as SETDB1 along with other chromatin modifying enzymes (Rowe et al., 2010; Schultz et al., 2002). Because URI and Art-27 involve potentially disparate mechanisms of gene repression through AR and KAP1, the objective of our study is to understand the role of the URI/Art-27 complex in regulating AR-mediated transcription and consequently its role in prostate cancer cell proliferation.

BODY

A description of the progress achieved for each specific aim is reported below:

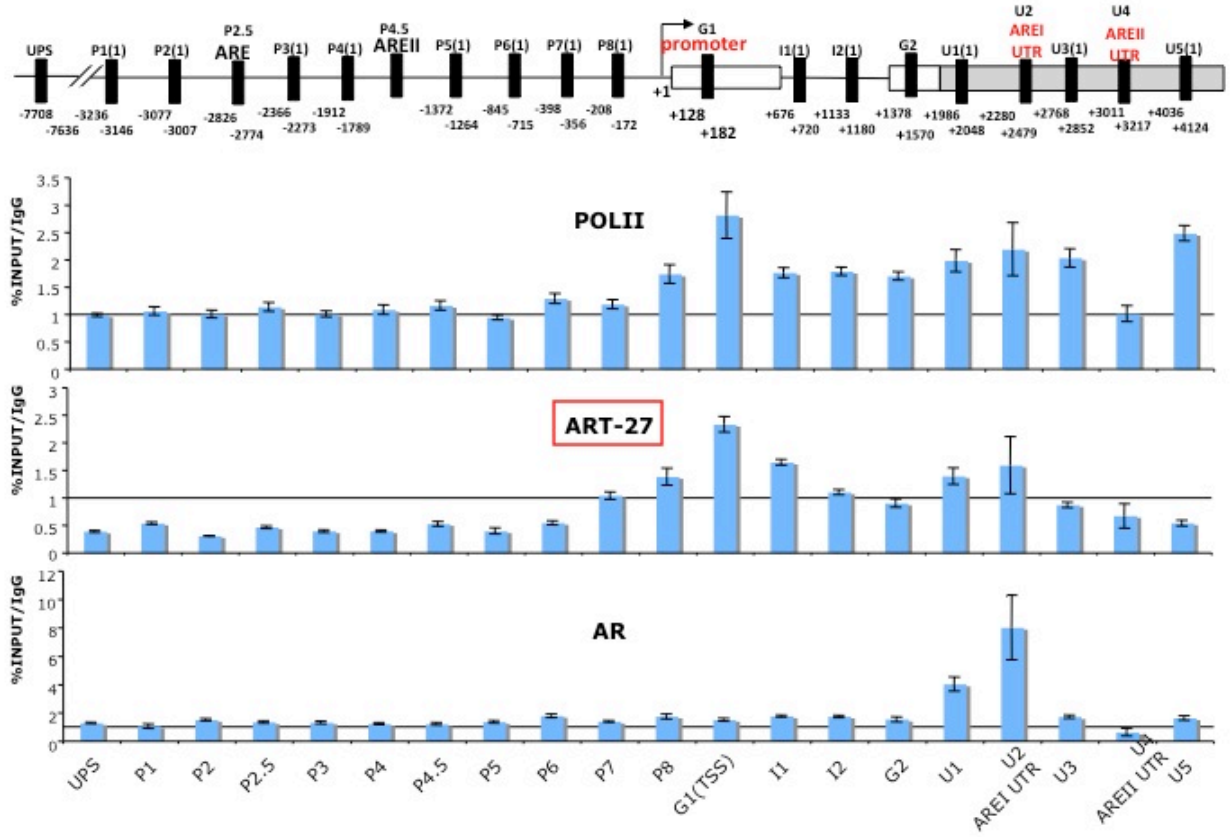
Specific Aim 1: Validate microarray findings.

Before the submission of the grant proposal we performed a genome-wide microarray analysis of androgen-regulated genes affected by URI knock-down. We examined whole genome expression in LNCaP cells treated with or without androgen and treated with a control siRNA or a siRNA directed against URI. This analysis allowed us to identify androgen-regulated genes modulated by URI. In the 2011 and 2012 reports, we validated several of these genes via qPCR. CONFAC analysis was then performed on the promoter regions of these genes to determine additional transcription factors (TFs) that potentially interact with URI to aid in its repressive or activating functions. Surprisingly, no statistically significant TF enrichment was found in genes requiring URI for repression. Among genes requiring URI for activation, the most enriched putative TF binding sites were for KLF4, E2F1. Oct1, NKX2.5, MYOD, HES1, FOXO4, GATA and POU1F1. Since the genes we identified and validated are all androgen-regulated, AR is also a TF requiring investigation.

Sub-Aim 1a: Validation of the transcription factor analysis by ChIP experiments.

I. Design and purchase primers specific for the promoter of the chosen genes
II. Test and titrate antibodies against the identified transcription factors in immuno-precipitation experiments
III. Determine recruitment of the identified transcription factors on the chosen genes' promoter

We have previously published that knockdown of Art-27 results in increased expression of the AR target gene Nkx3.1 (Nwachukwu et al., 2009). To gain a better understanding of the role of the AR/Art-27/URI transcription complex in mediating gene transcription, we performed ChIP analysis in LNCaP cells to locate each of these factors along the promoter and coding region of Nkx3.1 (Mita et al., 2011).

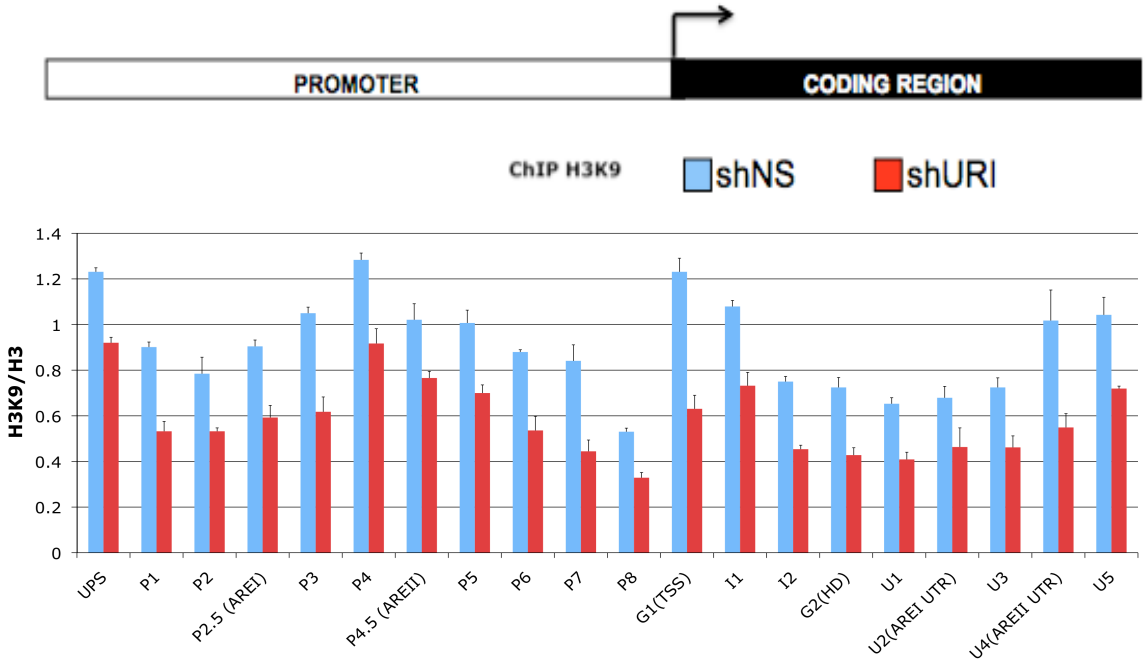


Using primers specific to the Nkx3.1 promoter and coding regions, we found that Art-27 is recruited to the transcription start site (TSS) along with RNA PolII. We were not able to ChIP URI using several commercial antibodies. Since URI associates with several subunits of RNA Polymerase in the cytoplasm (Boulon et al., 2010), it is likely that other proteins mask immunogenic epitopes while URI is in complex. Surprisingly, we also found that AR binds to ARE I in the coding region and not in the promoter. We have also demonstrated that URI depletion results in decreased Art-27 recruitment to the TSS and increased AR recruitment to ARE1 (Mita et al., 2011).

Transcription factors often recruit histone-modifying enzymes in order to facilitate or inhibit access to DNA by RNA polymerase, cofactors, and other TFs. In doing so, a TF can activate or repress gene transcription. Among other modifications, tri-methylation of histone 3 on lysine 9 (H3K9me3) in the promoter of a gene results in gene repression, while H3K9me3 in the coding region is consistent with gene activation (Li et al., 2007). Determining the class of histone marks present on repressed or activated genes can yield insight into which TFs, cofactors, and histone modification complexes are recruited or not recruited upon URI depletion.

Nkx3.1 is an AR target gene repressed by URI. Therefore, we hypothesized that URI depletion would reduce levels of the repressive H3K9me3 mark on both the promoter and coding region of Nkx3.1. To test this, we performed ChIP experiments to compare the relative amount of H3K9me3 on Nkx3.1 in cells depleted for URI versus control cells. LNCaP cells stably expressing an shURI or shNS (non-silencing) construct were hormone starved for 3 days and treated for 4 hours with 10nM DHT. Cells were then fixed and the chromatin processed for ChIP using a validated antibody for H3K9me3. qPCR was performed using a series of primers specific to the promoter and coding regions of Nkx3.1.

URI depletion results in decreased H3K9me3 on both the promoter and coding region of Nkx3.1, suggesting that URI plays a role in epigenetic regulation of AR target genes. Reduced H3K9me3 on the Nkx3.1 promoter is consistent with increased expression of Nkx3.1 upon URI knockdown. However, the corresponding decrease in the Nkx3.1 coding region suggests another regulatory mechanism is being overridden. Interestingly, the largest differences in H3K9me3 occur at the transcription start site (TSS), which has the highest accumulation of Art-27.



SETDB1 is a histone methyltransferase which mediates histone trimethylation (Schultz et al., 2002). In the 2012 report, Paolo demonstrated that URI binds to KAP1 and mediates its de-phosphorylation through PP2A. KAP1 de-phosphorylation then results in recruitment of SETDB1 to KAP1 target genes. It is possible that changes in histone methylation along AR target genes as a result of URI recruitment or depletion is due to differential recruitment of SETDB1 and possibly KAP1. Whether there is overlap between KAP1 and AR target genes remains to be investigated.

Specific Aim 2: Elucidate the role of ART-27 in the KAP1/URI complex

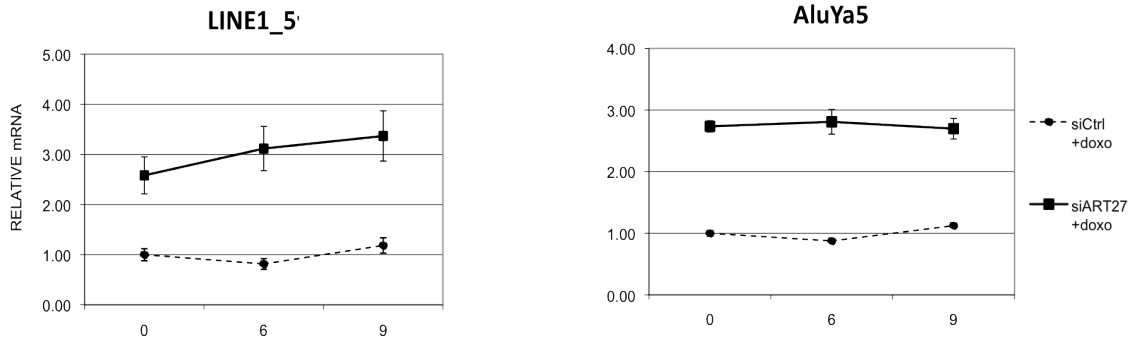
Mass spectrometry experiments with URI revealed a potential interaction with the transcription factor KAP1. This interaction was validated via immunoprecipitation in the 2012 report. URI depletion also resulted in increased expression of KAP1 target genes, which include retroelements such as LINE1 (Rowe et al., 2010). However, it is unknown whether the mechanism of Art-27-mediated gene repression acts through KAP1 through direct binding, or indirectly by regulating levels of URI. Therefore, to test for a possible direct interaction between KAP1 and Art-27, we performed immunoprecipitation experiments between Art-27 and KAP1 through overexpression in 293 cells.

Sub-Aim 2a: Validate a possible direct interaction between Art-27 and KAP1

i. Perform immunoprecipitation of endogenous KAP1 with ART-27 in both nuclear and cytoplasmic lysates of LNCaP prostate cancer cells
ii. Measure KAP1 target gene expression changes (NOXA, p21, BAX) upon siRNA knockdown of ART-27 in LNCaP cells

We transfected 293 cells to over-express either KAP1 alone, KAP1 + HA-tagged Art-27, or a vector control. We then performed immunoprecipitation (IP) using an anti-KAP1 antibody and blotted for HA. Indeed, we saw co-immunoprecipitation of HA-Art-27 with KAP1, revealing a direct interaction between Art-27 and KAP1.

Because we see a direct interaction between KAP1 and Art-27, we hypothesized that Art-27 knockdown would affect KAP1 target gene expression. We treated LNCaP cells with the DNA damage agent doxorubicin and transfected with either siCtrl non-silencing RNA or siART27.



Art-27 knockdown resulted in increased expression of LINE1 and AluYa5, a retroelement in the same class as LINE1. This provides more evidence for a link between Art-27 and KAP1-mediated gene repression.

Specific Aim 3: Evaluate the growth characteristics of stable ART-27 knockdown LNCaP cells
This aim was in progress last October when a hurricane struck NYU Langone Medical Center. The night of the storm, both regular and backup power was completely shut down to the entire medical center due to flooding of the basements of the buildings by the East River in New York City. All of the laboratories in the Medical Sciences Building where the Logan lab was housed were without any power and completely inaccessible for 48 hours. All of our stable cell lines along with most of our reagents were lost. The Medical Sciences Building has still not been restored. While we are temporarily housed in another building, it was not until March that we had access to tissue culture facilities and we did not have time to re-construct the stable cell lines necessary to complete this aim.

CONCLUSIONS

The androgen receptor is the key regulator of prostate cell growth, survival, and differentiation. Study of the mechanisms that regulate AR functionality are essential to understand the molecular steps that lead to prostate cancer and to develop new approaches to block prostate cancer cell growth. We previously demonstrated that URI, together with Art-27, is part of a novel AR repressor complex able to regulate AR-mediated transcription. In this report we find evidence that the co-repressor functions of Art-27 and URI are mediated through KAP1.

We demonstrated that URI knockdown resulted in increased H3K9me3 at the Nkx3.1 promoter using chromatin immunoprecipitation, suggesting that URI recruits histone methyltransferases as part of its co-repressor function. We also found that Art-27 is recruited to the transcription start site along with PolII, suggesting a potential mechanism for Art-27 to regulate transcription, while AR is present at an ARE in the coding region of Nkx3.1. In the 2012 report, we found that URI interacts with KAP1, which in turn can recruit the histone methyltransferase SETDB1 to repress transcription (Schultz et al., 2002). This might reveal novel crosstalk between KAP1 and AR, or suggest that AR interacts with other transcription factors requiring URI for transcriptional repression.

Finally, we demonstrated that Art-27 interacts directly with KAP1 and that knockdown of Art-27 causes de-repression of KAP1 target genes, suggesting that the repressive capacity of KAP1 is in part mediated by Art-27. Elucidating novel mechanisms of gene repression will help us understand how AR mediated genes are regulated in normal and cancerous prostate tissue.

KEY RESEARCH ACCOMPLISHMENTS

During the period covered by this report we:

1) determined via ChIP analysis that URI depletion results in reduced H3K9me3 on the Nkx3.1 promoter, consistent with a repressor function
2) determined via ChIP the binding sites of AR and Art-27 on the Nkx3.1 gene
3) demonstrated via immunoprecipitation a direct interaction between Art-27 and KAP1
4) demonstrated via qPCR that Art-27 knockdown results in increased expression of KAP1-regulated retroelements

REPORTABLE OUTCOMES

The work funded by this training grant has resulted in;

1) a presentation titled, “The AR repressor URI binds Art-27 and KAP1 to regulate gene expression” at the Nuclear Receptors & Disease 2012 meeting at Cold Spring Harbor Laboratory
2) a poster titled, “Regulation of Androgen Receptor mediated gene repression by URI” presented at the 9th annual NYU Cancer Center retreat (2012).
3) a “Works in Progress” presentation titled, “The AR co-repressor Art-27 regulates development” for the department of Pathology, 2013.

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